

APPLICATION FOR A UNITED STATES PATENT

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Title: LAYERED ALIGNED POLYMER STRUCTURES AND METHODS
OF MAKING SAME

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CROSS REFERENCES TO RELATED APPLICATIONS

This application is a continuation-in-part of co-pending Application No. 10/306,825, filed on November 27, 2002, which claims benefit of U.S. Provisional Application No. 60/337,286 filed on November 30, 2001, the entire contents of which
5 are incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

Accurate control of the orientation of polymeric structure in thin layers is desired to maximize their mechanical, chemical, and optical properties. While orientation can be performed through mechanical means, it is often more desirable to orient the structure during the polymerization process, particularly if the process involves
10 polymerizing the system into a specific final shape, where further mechanical manipulation is unfeasible.

Biomedical components often require oriented structures. Tendons, for example, contain highly oriented collagen fibrils, and the spinal intervertebral disc is composed mainly of oriented crystalline collagen fibrils and amorphous hydrophilic
15 proteoglycan. The prevalence of oriented collagen in the human body makes formation of highly oriented layers of this polymer a desired goal in modeling of tissue structure. Collagen is a biopolymer and protein, and found in the structure of tendons, skin, bones and blood vessels. Randomly oriented collagen materials are weak, and degrade quickly when exposed to mechanical stress. Soluble collagen is derived from animal tissue, and
20 can be obtained in a monomeric form. The collagen monomer will polymerize, termed “fibrillogenesis,” to form a gel-like structure. This process is achieved when the collagen is in a solution of specific pH, temperature, and ionic strength. The polymerization occurs as a self-assembly process, producing native-type collagen fibers. The fibers grow into a porous gel matrix.

25 The collagen molecule is a rod-like structure in the unaggregated state, composed of three peptide chains intertwined to form a triple helix. Improvements over randomly oriented gels in mechanical and optical properties can be realized if the aggregated molecules can be coerced into an oriented, or aligned, state. Cast films of

collagen can form a randomly oriented gel structure, which lacks desired mechanical and optical properties. It is desirable to give a structured order for optical transparency in corneal replacements.

Collagen fiber orientation techniques have included mechanical deformation of
5 already gelled matrices and laminar flow of a polymerizing matrix. These approaches yield gelled layers hundreds of microns thick.

SUMMARY OF THE INVENTION

The preferred embodiments of the present invention are directed at making templated extracellular matrix which can be used to generate full thickness corneal
10 stroma or other structured connective tissues. In a preferred embodiment, the invention provides a method of producing a corneal stroma comprising the steps of providing a nanostructured artificial template (NAT), contacting the NAT with a population of fibroblastic cells to produce a templated extracellular matrix, and stacking a plurality of templated extracellular matrices.

15 In other preferred embodiments, the invention provides a method of producing a templated extracellular matrix, comprising the steps of controlling a flow of a polymer solution into a device having a substrate, the device generating a shear flow to induce alignment of polymer structures; controlling a plurality of parameters during polymerization; generating a first layer of nanostructured artificial template; contacting
20 the first layer of nanostructured artificial template with a first population of cells; and maintaining the nanostructured artificial template and the first population of cells in a culture to produce a templated extracellular matrix. In another preferred embodiment, the invention provides a method of producing a templated extracellular matrix, comprising the steps of providing a nanostructured artificial template; contacting the
25 nanostructured artificial template with a first population of cells; and maintaining the nanostructured artificial template and the first population of cells in culture to produce a templated extracellular matrix having a first surface and a second surface.

In other preferred embodiments, the invention provides a biomimetic corneal stroma produced by the steps of providing a nanostructured artificial template; contacting the nanostructured artificial template with a first population of eukaryotic cells; maintaining the nanostructured artificial template and the first population of cell 5 in culture to produce a templated extracellular matrix; repeating the steps of providing, contacting and maintaining to produce additional templated extracellular matrices; and stacking a plurality of templated extracellular matrices oriented at any arbitrary angle with respect to each other. Typically the eukaryotic cells are mammalian fibroblasts, preferably human keratocytes. In preferred embodiments, the eukaryotic cells are 10 treated with an ascorbate compound to activate secretion of extracellular matrix. The eukaryotic cells can be activated before or after coming into contact with NAT. In preferred embodiments, activated cells are made quiescent by removal of the ascorbate compound and preferably additionally contacting the cells with fetal bovine serum.

In a preferred embodiment, the present invention provides a biomimetic 15 cornea produced by the steps of providing a nanostructured artificial template; contacting the nanostructured artificial template with a first population of eukaryotic cell; maintaining the nanostructured artificial template with the eukaryotic cells to form a template extracellular matrix; repeating the steps of providing, contacting and maintaining to form additional templated extracellular 20 matrices; stacking a plurality of templated extracellular matrices oriented at any arbitrary angle with respect to one another to form a multilaminar templated extracellular matrix; contacting a first surface of the multilaminar templated extracellular matrix with a second population of cells; and maintaining the multilaminar templated extracellular matrix in culture to form a biomimetic 25 cornea.

In other embodiments, the invention provides a method of producing a nanostructured artificial template comprising one or more thin, oriented layers of polymer material. The material is preferably produced by the method of introducing a shearing-flow to a free surface in a predominantly monomeric solution of the self-

assembling polymer sub-units, and inducing polymerization or growth of the monomer while in this shearing flow. The system for forming the oriented layer of material provides relative movement between a delivery system and the substrate on or over which the material is deposited. In general, any method to produce shear, elongation, or 5 parabolic flows between substrates may be used. The rate of flow of the material from the delivery system and/or the relative velocity between the deposition surface and the material as it is delivered to the surface are controlled to properly orient the material at the desired thickness. These rates can be adjusted to vary the properties of the film in a controlled manner. Preferred embodiments include either angular or linear relative 10 movement between the delivery system and the substrate.

It should be noted that deposition of the polymer solution can occur on a substrate having curved or spherical surfaces to result in stress-free interlayer boundaries. This embodiment is enabling for corneal constructs.

In a preferred embodiment, the step of controlling the temperature, pH, solvent 15 chemistry, and relative humidity during the polymerization process is performed on a local level.

The preferred embodiment of the present invention further comprises a layered construct composed of layers of sub-micron to 10 µm thick oriented polymeric films or fibers, with the principle direction of orientation alternating in each subsequent layer. A 20 preferred embodiment of the invention uses this method to form synthetic biocompatible or biopolymeric materials such as implantable tissue material. These materials can be implanted as soft tissue replacement or for bone or joint replacement or repair.

In another preferred embodiment of the present invention, the method further 25 includes inducing fibrillogenesis of the collagen while in this shearing flow. The method further includes controlling the collagen monomer concentration, temperature, solution properties and relative humidity of the fibrillogenesis process, producing collagen material having an oriented fibrillar structure in a sheet with a uniform, controllable thickness. The thickness can range from approximately 500 nm to 100 µm.

In accordance with another preferred embodiment, the method for producing a multi-layer construct can be used to form an artificial corneal construct. The collagen layers can be seeded with endothelial and epithelial cells, which generate a negative pressure field in the construct, and compress the construct to a thickness necessary for optical transparency.

A preferred embodiment includes a method of producing a thin film of oriented polymer structures, including the steps of controlling the flow of a polymer solution into a device having a substrate, the device generating a shear flow to induce alignment of polymer structures, controlling a plurality of parameters during polymerization; and generating a layer of oriented polymer. The method further includes the polymer being a biopolymer such as collagen. The method further comprises the steps of mixing a solution of collagen with phosphate buffered saline solution, adjusting the pH of the solution to 7.4 ± 0.2 , applying the solution at a controlled rate onto a substrate which generates a shearing flow, causing preferential orientation of the gelling collagen fibrils; and generating successive layers, each layer representing a portion of the component. The method layers have a uniform, controllable thickness ranging from sub-micron to 100 microns. The collagen is either type I or type V collagen. The principle orientation of the aligned fibrils in a single layer alternates in each successive layer. The angle between the principle orientation of each layer is approximately in the range of 0 to <90 degrees. The solution properties, including temperature, concentration and surfactant composition are controlled. The shear flow is generated by spinning the substrate at a controlled rate in a range of approximately 50 to 50,000 Hz.

In other embodiments, the present invention provides a method of making a multilaminar nanostructured template comprising introducing a monomer solution from a first inlet, between a polymer accepting surface and a polymer rejecting surface to first outlet to produce an aligned polymer layer; increasing the spacing between the polymer accepting surface and the polymer rejecting surface; introducing the monomer solution into a second inlet and recovering the monomer solution from a second outlet wherein the flow from the second inlet to the second outlet is substantially orthogonal to the

flow from the first inlet to the first outlet; and producing an aligned polymer layer in which the polymer molecules are substantially orthogonal to the polymer molecules of the previous layer.

In a preferred embodiment the present invention provides a method to generate

- 5 single or multiple layers of aligned polymer fibrils by pumping monomer solution between two surfaces, with an adjustable gap between them. One of the surfaces is collagen accepting and the other surface is collagen rejecting. Single or multiple layers of collagen may be produced by creating collagen self assembly conditions within the gap between the surfaces. A collagen accepting surface may be generated by coating the
- 10 surface may be generated by coating the surface with antibodies to collagen, by plasma cleaning, by cleaning with micro 90, by functionalization, or by treating the surface with any methods known to attract and promote adherence of collagen monomer or polymer units. A collagen rejecting surface may be generated by functionalization, surface treatments, coatings or use of materials known to limit, reduce, or reject the adhesion of
- 15 collagen monomer or polymer units.

This invention further provides a method of producing a nanostructured artificial template that is useful for directing the production of extracellular matrix by eukaryotic cells such as fibroblasts. In general, the nanostructured artificial template is an aligned array of linear structures sufficient to induce a fibroblast to produce an aligned

- 20 extracellular matrix which can in turn iteratively act to induce fibroblasts to produce subsequent aligned extracellular matrix. In preferred embodiments, the aggregate extracellular matrix product of the population of fibroblasts is an aligned nanostructured array that functions as a template for further extracellular matrix production by fibroblasts. In preferred embodiments, the aligned extracellular matrix array produced
- 25 by the population of fibroblasts in response to the nanostructured artificial template is oriented with respect to the linear structures of the nanostructured artificial template. In preferred embodiments, each successive aligned extracellular matrix array are rotated some angle, theta, with respect to the orientation of the previous template, where theta has a value from about zero to about 90 degrees.

The foregoing and other features and advantages of the system and method for producing a multilayer construct of sub-micron to 10 micron thick layers of oriented collagen fibers will be apparent from the following more particular description of preferred embodiments of the system and method as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a preferred embodiment of the apparatus used to generate a single layer of oriented self-assembled polymer molecules in accordance with the present invention.

Figure 2 illustrates a flow chart for producing aligned collagen fibers in accordance with a preferred embodiment of the present invention.

Figures 3A and 3B illustrate electron micrographs of collagen fibers prepared by a spin-coating method where Figure 3A particularly illustrates random alignment of fibers and Figure 3B illustrates fibers aligned in flow field in accordance with preferred embodiments of the present invention.

Figures 4A and 4B illustrate a preferred embodiment of the apparatus used to generate a matrix of orthogonally-aligned layers in accordance with the present invention.

Figures 5A-5C illustrate another preferred embodiment of an apparatus used to generate a matrix of orthogonally-aligned layers in accordance with the present invention.

Figures 6A-6C illustrate schematically a folding method to generate a multilayer structure with orthogonally-aligned layers in accordance with a preferred embodiment of the present invention.

Figure 7 schematically illustrates collagen fibers in the lamellae of the stroma in accordance with a preferred embodiment of the present invention.

Figure 8A illustrates an apparatus having an offset holder wherein a monomer solution is deposited onto the substrate at the center of the offset disk holder in accordance with a preferred embodiment of the present invention.

Figure 8B illustrates a flow chart of a preferred embodiment for producing
5 aligned collagen fibers in accordance with a preferred embodiment of the present invention.

Figure 9 is a scanning electron microscope (SEM) image demonstrating the deposition of a plurality of thin aligned layers onto a glass substrate in accordance with a preferred embodiment of the present invention.

10 Figure 10 is a scanning electron microscope (SEM) image of a single layer of predominately aligned collagen fibrils in accordance with a preferred embodiment of the present invention.

Figure 11 is a scanning electron microscope (SEM) image of aligned collagen fibrils generated by the spin-coating methodology in accordance with a preferred
15 embodiment of the present invention.

Figure 12 illustrates a scanning electron microscope (SEM) image demonstrating layering of collagen in pseudolamellae in accordance with a preferred embodiment of the present invention.

Figure 13 is a scanning electron microscope (SEM) image illustrating the
20 interaction of two individual layers of aligned collagen in accordance with a preferred embodiment of the present invention.

Figures 14A-14C schematically illustrates a nanofabrication system and a flow-focussing method to manufacture layered, aligned polymer structures in accordance with a preferred embodiment of the present invention.

25 Figure 15 schematically illustrates another preferred system to manufacture layered, aligned polymer structures in accordance with a preferred embodiment of the present invention.

Figures 16A and 16B illustrate a block diagram and a schematic diagram, respectively, of a preferred embodiment system to manufacture layered, aligned polymer structures in accordance with a preferred embodiment of the present invention.

Figure 17A is a graphical representation of the relationships between positions 5 within the layer or layer thickness on the ordinate and radial position and/or velocity on the abscissa for conditions of 750 rpm (dashed lines) and 1600 rpm (continuous lines) with constant addition of collagen solution (0.1 ml/min) to the center of a 2.5 cm radius disk in accordance with a preferred embodiment of present invention.

Figure 17B is a graphical representation 520 of the relationships between shear 10 rate or velocity of fluid or the ordinate and normalized position in layer at 25 mm from center on the abscissa for conditions of 750 rpm (dashed lines) and 1600 rpm (continuous lines) in accordance with a preferred embodiment of present invention.

Figure 18 is a scanning electron microscope (SEM) image illustrating the interaction of two individual layers of aligned collagen in accordance with a preferred 15 embodiment of the present invention. The image shows fibril alignment 2.0 cm from the fluid deposition point; with a flow rate of 0.25 ml/min and the rotation velocity of 1600 rpm. The top layer in this particular area formed incompletely due to de-wetting during the film formation, and the lower, orthogonal layer of fibrils can be seen passing under the top layer.

Figure 19 schematically illustrates an Ussing-style perfusion chamber and a 20 nanostructured artificial template (NAT) comprising collagen placed in between the half-chambers with a pressure applied to apply strain to the structure in accordance with a preferred embodiment of present invention..

Figures 20A-20C illustrate the flow chart of a method for producing a templated 25 extracellular matrix comprising at least one population of cells from a nanostructured artificial template in accordance with a preferred embodiment of the present invention.

Figure 21A schematically illustrates a flow chamber for generating layered aligned polymer in accordance with a preferred embodiment of present invention.

Figure 21B is a top view of the flow chamber illustrated in Figure 21A.

DETAILED DESCRIPTION OF THE INVENTION

The systems and methods of the present invention relate to processes and resulting material, suitable for the guided production of the self-assembly of aligned polymers in layers, and structures having multiple, non-aligned layers. More particularly the systems and methods of the present invention relate to the production of 5 self-assembled biocompatible non-aligned thin lamellae.

This invention will be better understood with reference to the following definitions:

“Self-assembly”: a process by which a system spontaneously combines a number of smaller structural elements to form a large molecular or supramolecular complex.

10 Self-assembly is the process by which many biological complexes form, such as DNA and collagen. This reaction sometimes requires that a threshold temperature, solution condition, or pH is reached before the spontaneous reaction occurs.

“Gel”: a material that is crosslinked through bonds of sufficient strength that it cannot be dissolved in ambient conditions. The bonds can be covalent, ionic, physical, 15 or other.

“Stroma”: the supporting tissues or matrix of an organ, such as a connective tissue framework.

20 “Collagen”: a self-assembling biopolymer produced by reacting monomeric collagen in a neutral pH, slightly ionic solution. Collagen is a structural building block in the body, and is found in the cornea, skin, bones, blood vessels, ligaments, tendons, and cartilage.

“Polymerization”: the process by which monomer-sized molecules are assembled to form supramolecular structure. This process can occur through the formation of permanent chemical bonds, ionic bonds, or associative bonds.

25 Fibrillogenesis is a form of polymerization in collagen.

"Polymer": any supramolecular structure comprised of repeating subunits.

These structures can be naturally occurring, such as proteins, or man-made, such as polyolefins.

A preferred embodiment of the invention comprises a method to produce a
5 highly oriented, thin film of self-assembled polymer. The method comprises the process
of subjecting a solution of monomeric building blocks of the self-assembling polymer to
a shearing flow. While under the influence of this shearing flow, the polymer self-
assembles, forming oriented polymeric structure in a thin film from approximately 500
nm to 100 μm thick. The preferred embodiments of the present invention provide for
10 several processes of generating this shearing flow, examples of which are described
herein below. The systems and methods of the present invention also provide for a
process of forming constructs with multiple layers of aligned polymer structures, each
layer having a different principle direction of orientation. The preferred embodiments
provide for a method of forming constructs in both biopolymers and synthetic polymers.

15 **Example 1**

As one example of a preferred embodiment of this present invention, a metal
disk is used as a deposition substrate. The disk is mounted on a spin-coating-type
apparatus. A chamber is built around the apparatus to control the temperature and the
relative humidity. The disk can be spun at a specified rate. This embodiment is
20 illustrated in Figure 1.

A solution of Vitrogen collagen is chilled to 4-6 °C. Eight ml of the collagen
solution is mixed with 1 ml of 10X phosphate-buffered saline solution (0.2 M
Na₂HPO₄, 1.3M NaCl, pH=7.4) and 1 ml of 0.1M NaOH. The pH is adjusted to 7.4 ±
0.2 by adding 0.1M HCl. The solution is warmed to the test temperature, then steadily
25 dripped onto the rotating substrate 16. The collagen gels form a uniform sub-micron
thick sheet. This process is described with respect to the flow chart illustrated in Figure
2. Nematic stacks are prepared by cutting out sections of the radially-aligned collagen
fiber sheets, and stacking them orthogonally by hand. Electron micrographs of

randomly oriented collagen fibers and oriented collagen fibers in a single layer prepared by this method are shown in Figures 3A and 3B, respectively.

5 **Example 2**

As a second example of a preferred embodiment of the present invention, sample disks 104 with T-slots cut into their surfaces are mounted on a disk 102 rotating at a specified rate as shown in Figures 4A and 4B. In accordance with a preferred embodiment of the present invention, the first layer of aligned associating fibrils is 10 prepared by flowing the polymeric solution into channel of sample disks, which are on the rotating bottom disk. The centrifugal motion generates a shearing flow, which produces a thin layer and aids in aligning the growing associating polymers. The sample disks are then rotated 90 degrees, or to another specified angle, and the second layer is applied, which aligns with the specified angle relative to the first. A solution of 15 Vitrogen collagen is chilled to 4-6 °C. Eight ml of the collagen solution is mixed with 1 ml of 10X phosphate-buffered saline solution (0.2 M Na₂HPO₄, 1.3M NaCl, pH=7.4) and 1 ml of 0.1M NaOH. The pH is adjusted to 7.4 ± 0.2 by adding 0.1M HCl. The solution is warmed to the test temperature, then steadily dripped onto the center of the rotating disk, so that the solution travels down the channels extending to the outer radius 20 of the rotating disk. After the first layer has gelled into an oriented collagen film, the sample disks are rotated 90 degrees, and the second layer is applied, so that the orientation direction of the second layer is 90 degrees with respect to the first layer. This process is repeated many times to generate a matrix of orthogonally-oriented collagen fibril layers.

25 **Example 3**

Another preferred embodiment of the present invention includes the monomer solution being placed in a temperature-controlled bowl as shown in Figures 5A-5C. In Figure 5A, the polymer solution is subjected to a shearing flow by rotating the ball in one direction, or by oscillating in the same rotation plane. The polymer associates

during this process. In Figure 5B, the ball is rotated or oscillated in the orthogonal plane, to generate a layer of associated polymers orthogonal to the first layer. In Figure 5C a three-dimensional (3D) rendering of the method is illustrated. A ball 122 with a diameter a few microns smaller than the bowl 128 diameter is placed in the bowl. A shaft 126 attached to the ball rotates the ball first in one direction, generating a shearing flow, during which time the monomer polymerizes. After a designated gelation period, fresh monomer solution may be introduced to the bowl, and the ball is rotated in the orthogonal direction, creating a layer orthogonally-aligned to the first layer.

Example 4

As another embodiment of the material described in this invention, highly oriented collagen layers prepared as described herein can be bundled to form the annulus fibrosus found in the spinal intervertebral disk. This disk acts primarily as a weight-bearing and flexible joint. The load bearing capability and flexibility in selected directions is achieved by the combination of the annulus fibrosus and nucleus pulposus.

Annulus fibrosus is a layered structure that is rigid in the radial direction but deformable in the axial direction and by torque. This structure has alternating layers of oriented collagen fibrils, similar to that described in the cornea. Each layer has its collagen fibrils wound at an angle, and subsequent layers have an alternate orientation. Such a structure helps to achieve a maximum resistance to radial stress, while allowing a deformation in torque and bending.

Example 5

As another preferred embodiment of this invention, single layers of aligned polymer structure are generated with the techniques and methods discussed herein. The layers are folded as shown in Figures 6A-6C, to generate successively crossed layers of aligned polymer fibrils or other superstructure.

The extracellular matrix of the mature intact cornea comprises an extremely varied yet highly structured array of collagens, proteoglycans, glycoproteins and soluble macromolecules.

Current attempts to generate a biomimetic corneal construct have yielded corneas that behave similarly to *in vivo* corneas with respect to the function of the cellular layers. However, these corneal constructs have severe limitations with regard to the structure of the stromal extracellular matrix (ECM), which was constructed to ensure biocompatibility, nominal transmittance and the ability to promote adherence of the superficial cell layers and little more. It appears that previous investigators focused on the cellular layers but lost sight of the importance of the stroma itself. The three major functions of the cornea: protection, refraction and transmission, are performed primarily by the stromal ECM, the structure of which is optimally designed to accomplish these objectives. Thus, the cellular layers serve only to maintain and defend the stroma, which provides the principle functions of the cornea:

Strength. The major structural collagen of the stroma (type I collagen) is arranged in 300 to 500 lamellae of parallel, non-crosslinked fibrils. The lamellae are stacked in the anterior posterior (AP) direction and the fibrils of adjacent lamellae are nearly perpendicular to each other. Free ends of the fibrils have not been discerned in the cornea, which suggests that they run uninterrupted from limbus-to-limbus. This "plywood" arrangement of the lamellae gives the cornea remarkable strength tangential to the surface. Randomly crosslinked collagen networks of similar thickness (used in current constructs) cannot provide similar yield strength.

Refraction. The ability of the fibrils to slide relative to each other with ease allows the natural cornea to distribute the load imposed by the intraocular pressure (IOP) uniformly. This enables the anterior surface of the eye to form a nearly perfectly spherical shape for refraction. Stromas comprising randomly cross-linked collagen are likely to form imperfect surfaces for refraction upon inflation to normal IOP. This is due to the complex stress fields that would be produced when they are loaded.

Transmission of light. The uniform diameter of natural corneal fibrils and their short-range ordering allows light to pass through virtually unimpeded. Randomly crosslinked collagen networks, though nominally transparent, cannot produce the same quality optical properties. The process described in the embodiments of this invention

can produce orthogonally stacked arrays of aligned type I/type V collagen fibrils. The general biomechanical and optical properties of such a construct should be similar to native corneal stroma.

The difficulties in forming a fully complete, functional, corneal stroma,

- 5 constructed *de novo*, by artificial means have led some to indicate that it may not be a viable given the technology available today. However, it is not likely to be necessary to complete the construction of the stroma purely by mechano/chemical manipulation of biochemical components in the laboratory. A partially completed primary corneal stroma comprising orthogonal layers of aligned type I/type V heterotypic collagen fibers
10 can suffice as a suitable scaffolding or starting point, as it does during embryogenesis. In support of this approach, it is known that following full-thickness trephination wounds in rabbit corneas, the healing response is capable of transforming the initially opaque fibrous plug to tissue that is similar to normal corneal stroma. The initially opaque scars comprise large interfibrillar spaces, unusually large chondroitin sulfate
15 (CS) proteoglycans, hyaluronic acid and no detectable keratan sulfate proteoglycan. After one year of healing and remodeling, normal interfibrillar spacing, size and distribution of proteoglycans is restored. This mechanism works even if tissue of similar biochemistry, but not structure is implanted into the cornea. It has been found that the corneal healing response has the capacity to partially resolve collagen fibril
20 structure even when scleral tissue is used to repair corneal wounds (Winkelman 1951 Am J Ophthalmol; Kurz 1953 Cs Oftal; Maurice and Singh Cornea 1996) incorporated herein by reference in its entirety. The present invention takes advantage of the natural healing response and remodeling ability of the corneal stroma. A stromal scaffolding remodeled by the corneal wound healing response, can be implanted. However,
25 implanting a biomimetic stroma that is not sufficiently strong, transparent, or smooth at the outset is not viable. The artificial stroma, at the outset, must preserve the optical qualities of the cornea during the remodeling period. Current corneal constructs, which employ randomly cross-linked type 1 collagen fibrils, cannot meet this requirement.

The closer the tissue or scaffolding to be remodeled mimics native stroma, the less time it takes to fully resolve the structure and fully incorporate the graft. Further, if the initial scaffolding is capable of performing the three major functions of the stroma (protection, refraction and transmission), remodeling *in vivo* may proceed while vision
5 remains clinically acceptable.

Thus, from investigations into the developmental biology of the chicken, it has been learned that corneal embryogenesis is an intricately orchestrated and complex phenomenon. Initially, from superficial epithelial cells derived from an offshoot of the developing brain, an orthogonally organized primary corneal stroma of type I collagen is
10 secreted layer-by-layer. The extracellular matrix (ECM) of the mature intact cornea comprises an extremely varied yet highly structured array of collagens, proteoglycans, glycoproteins and soluble macromolecules.

The three major functions of the cornea: protection, refraction and transmission, are performed primarily by the stromal ECM, the structure of which is optimal to
15 accomplish these objectives. Thus, the cellular layers serve only to maintain and defend the stroma. In addition, the major structural collagen of the stroma (type I collagen) is arranged in approximately 300 lamellae of parallel, non-crosslinked fibrils. The lamellae are stacked in the anterior posterior (AP) direction and the fibrils of adjacent lamellae are nearly perpendicular to each other. Free ends of the fibrils have not been
20 discerned in the cornea, which suggests that they run uninterrupted from limbus-to-limbus. This "plywood" arrangement of the lamellae gives the cornea remarkable strength in tension tangential to the surface. Randomly crosslinked collagen networks of similar thickness (used in current constructs) cannot provide similar yield strength.

The ability of the fibrils to slide relative to each other with ease, allows the
25 natural cornea to distribute the load imposed by the intraocular pressure (IOP). This enables the anterior surface of the eye to form a nearly perfectly spherical shape for refraction. Stromas comprising randomly cross-linked collagen are likely to form imperfect surfaces for refraction upon inflation to normal IOP. When these random

networks are loaded, complex stress fields are formed that result in inhomogeneous refraction and thus compromised optical qualities.

The uniform diameter of natural corneal fibrils and their short-range ordering allows light to pass through the cornea virtually unimpeded. Randomly crosslinked 5 collagen networks, though nominally transparent, cannot produce the same quality optical properties because of the varying spacing in the random network.

With regard to biomimetic corneal constructs, recent attempts have been made to develop full corneal constructs de novo. These efforts have yielded corneas that behave similarly to in vivo corneas with respect to the function of the cellular layers. However, 10 both of these corneal constructs have severe limitations with regard to the structure of the stromal ECM, which was constructed to ensure biocompatibility, adequate transmittance and the ability to promote adherence of the superficial cell layers and little more.

Completely artificial corneal replacements, such as the K-pro, have not met with 15 widespread clinical success. Such devices are not yet qualified for use in clinical situations where a transplant would be considered even marginally effective because of the high potential for devastating complications and the need for continual high quality follow-up. Current incarnations of corneal constructs generated from biomimetic materials and live cell layers do not include physiologically, ultrastructurally or 20 biochemically realistic stromas. In addition there have been a number of efforts towards the production of an artificial cornea which have not met with complete success.

With regard to effective scaffolding and wound healing response, a fully 25 complete, functional, corneal stroma, constructed de novo, by artificial means may not be a viable approach given the technology available today. However, it is not likely to be necessary to complete the construction of the stroma purely by mechano/chemical manipulation of biochemical components in the laboratory. A partially completed primary corneal stroma comprising orthogonal layers of aligned type I/type V heterotypic collagen fibers might suffice as a suitable scaffolding or starting point, as it does during embryogenesis. Such a partial solution would be a marked improvement

over current systems, allowing easy cell infiltration and critically, immediate functionality both optically and mechanically. In support of this approach, it is known that following full-thickness trephination wounds in rabbit corneas, the healing response is capable of transforming the initial opaque fibrous plug/scar to tissue that is similar to 5 normal corneal stroma. The initially opaque scars comprise large interfibrillar spaces, unusually large chondroitin sulfate (CS) proteoglycans, hyaluronic acid and no detectable keratan sulfate proteoglycan. After one year of healing and remodeling, normal interfibrillar spacing, size and distribution of proteoglycans is restored. This mechanism works even if tissue of similar biochemistry, but not structure is implanted 10 into the cornea. It has been found that the corneal healing response has the capacity to partially resolve collagen fibril structure even when scleral tissue is used to repair corneal wounds. This suggests that it might be possible to take advantage of the natural healing response and remodeling ability of the corneal stroma. Stromal scaffolding, which can be remodeled by the corneal wound healing response, can be implanted.

15 However, implanting a biomimetic stroma that is not sufficiently strong, transparent, or smooth is not acceptable. The artificial stroma, at the outset, has to preserve the optical qualities of the cornea during the remodeling period. Current corneal constructs, which employ randomly cross-linked type I collagen fibrils, cannot meet this requirement.

Figure 7 illustrates collagen fibers in the lamellae of the stroma in accordance 20 with a preferred embodiment of the present invention. The behavior of associate rods (collagen) in solution is explored. Collagen fibrils are composed of triple-helix collagen macromolecules that are approximately 300 nm long and 1.5 nm in diameter. These segments are produced inside cells and are excreted as procollagen whereupon the ends are removed to form a macromolecule that naturally self-assembles. Although there is 25 some debate over the exact mechanism of this self-assembly process, it is understood that the assembly of collagen macromolecules into fibrils is an entropy driven process. The most favorable structure is therefore a cylinder 184 which minimizes the surface area. This fibril assembly is very similar to other protein polymerizations. Each of these cylindrical bundles is composed of segments of associated molecules in the

familiar 67 nm repeating structure. There is still some debate over the exact molecular process of association but it appears that the cylindrical bundles of segments end-associate whereby the terminating C-telopeptide 192 associates with the N-telopeptide on the adjacent chain. The telopeptides also influence the diameter of the fibril and the 5 segment packing, but there are also probably influences from glycosaminoglycans (GAGs), proteoglycans, solution temperature and concentration. All of these components can be controllable in a manufacturing process. Further, there is some evidence that the association is driven by a further hydrophobic effect between the telopeptides. These fibrils then associate to form fibers.

10 Thus with no other influence, a solution composed of collagen macromolecules (*i.e.* the basic triple helix unit) forms an isotropic random gel. However, with the correct driving force, these gels can be forced to grow in an aligned manner. Magnetism, drainage flows and confining effects have both been used successfully, but only with weak influence on the morphology of the resulting gels.

15 Orientation of a rigid-rod in a flow field is determined by the balance of hydrodynamic forces (aligning) and rotary Brownian motion (randomizing) which is described by the Peclet number $Pe = II_{2D}/D_r$, where D_r is the Brownian diffusion coefficient and II_{2D} is the convective diffusion coefficient. Simple estimates suggest that the flow field can orient the collagen monomers because of their size ($Pe < 1$) but 20 that in fact for the monomers the flow is very close to the transition ranges. However, as the monomers associate and the collagen fibrils grow, the aspect ratio increases and consequently the Peclet number increases rapidly as it is related to the cube of the length of the rod.

$$Pe = \frac{II_{2D}^{1/2}}{D_r} = \frac{\dot{\gamma}}{D_r} \quad \text{Equation 1}$$

25

$$= \frac{8\pi\eta_s a^3 \dot{\gamma}}{3kT(1n(2r_p) - \frac{1}{2})} \text{ for thin prolate spheroids } (r_p \gg 1) \text{ in shear flow,} \quad \text{Equation 2}$$

wherein $r_p = \frac{a}{b}$ is the aspect ratio of spheroid (a is long dimension). At 300 K for rigid rods of length 300 nm and diameter 30 nm in a solvent of 1 mPa.s and at a shear rate of 600 s⁻¹ the Peclet number is 13.

In addition, the proximity of walls also act to orient the monomers in the plane of the wall. This influence is especially strong in the confined films which are only of an order of magnitude larger than the longest axis of the collagen monomers.

Concentrated solutions of rigid non-interacting rods are known to behave in a liquid crystalline fashion, forming nematic structures where there is long range order in the direction of the long axis of the rod. In theory, the direction of this ordering is arbitrary

but is usually generated by perturbations, such as the confining effect of a wall, or solvent flow. Although nematic structures built from non-interacting rods require a critical concentration to transition from an isotropic to a nematic phase. In the case of interacting rods, the situation is more complex, but the interactions can clearly influence the ability of the molecules to align. Once fibrils have begun to grow the flowing monomers passing across the substrate in the shear field naturally end attach since there is a pronounced anisotropy between side addition and end addition for interacting rods. In addition magnetic (or electric) fields are known to strongly align nematic liquid crystals and have been proven to influence the alignment of growing collagen gels.

With regard to the alignment of polymers, it is well known that flow aligns polymers. Both shear and extensional flows align polymers in different manners but in general if the flow is strong enough there is some form of alignment in the direction of the flow. Spin-coating, and other similar flows, such as film drainage and steady shear, have been observed to produce roughly oriented polymers from the melt, for rigid-rod molecules and for liquid crystals. Alignment in these systems is often however only local and is usually for systems where the macromolecules themselves are being aligned. The alignment of growing fibers or polymers is less well recognized a process.

It has also been observed that shear induces alignment in polymers during the polymerization process of rod-like molecules. However simple shear does not provide sufficient force to counter the random fluctuations of the chains, and their natural

propensity move under Brownian motion. The preferred embodiments of the present invention use spin-coating methods to provide a flow regime suitable for initiation and growth of aligned collagen fibrils. The solution conditions can be modulated while the polymerization process is proceeding, and the naturally radial nature of the flow assists
5 in maintaining the fiber separation and promotes growth from a central core area. In addition the spin-coating process provides a natural length scale that can be easily adjusted to control the layer thickness and rate of polymerization. Also, since it is known that the proximity of a surface influences the position of an adjacent molecule, the thin confinement layer promotes alignment in the most critical direction, parallel to
10 the layers longest axis. Spin-coating has been used to produce thin homogeneous films for a number of years. In melts and liquid crystals this deposition process is known to align polymers. This method is extremely well understood for evaporating and non-evaporating solvents since it is used heavily commercially in silicon microfabrication processes and in the optics industry.

15 With regard to the alignment of collagen, examples of artificial alignment of collagen are relatively common in the literature, but rarely result in the highly aligned configuration that is required for a truly biomimetic system. Previously noted parameters such as gravity, shear, diffusion and other external forces can influence the morphology of the fibers, but in all cases the alignment is weak. Other observations
20 include alignment of collagen-like self-assemblies using shear while cooling from a hot solution. All of these cases require relatively concentrated solutions which limit the control over the fiber morphology. In addition collagen has been aligned using the natural propensity of collagen monomers to self-assemble in concentrated solutions. However, this method allows no control of the fiber sizes and shapes. To provide the
25 highly aligned morphology two things are critically required, a thin confinement layer, and a unidirectional flow. Spin-coating provides both requirements readily. Although spin-coating has been observed to generate alignment in thin films, these are usually from melts and in polymerized systems. It should be noted that alignment as

traditionally detected for many of these systems, using Circular Dichroism, does not indicate alignment on the levels required by a corneal analog.

Figure 8A illustrates an apparatus having an offset holder wherein a monomer solution is deposited onto the substrate at the center of the offset dish holder in accordance with a preferred embodiment of the present invention. Figure 8B illustrates a preferred methodology to create single and multiple layers of aligned polymer films in accordance with a preferred embodiment of the present invention. In this system and method a substrate is placed off-center on a substrate holder. The holder 206 is rotated at a prescribed velocity or sequence of velocities to create a thin shearing flow. The monomer solution 202 is added to the deposition substrate 210 (either by steady flow or by unsteady dripping) and is carried radially away from the injection point by the centripetal acceleration. Thus a single layer (film) of aligned collagen fibrils is created. To generate multiple layers with different alignment directions, the deposition substrate 210 is rotated at any prescribed angle and another layer may be deposited.

Figure 8B is a flow chart describing method 216 for generating aligned collagen via spin-coating in accordance with a preferred embodiment of the present invention. The method begins with step 218 of obtaining commercially available type I collagen monomers. They are kept refrigerated at 4-6°C. Extracted or recombinant human and/or animal collagen type I monomers may be used. Collagen type I monomer concentrations are in the range of 0.01 mg/ml to 100 mg/ml with a preferred range of 0.5 to 10 mg/ml. A preferred embodiment utilizes Vitrogen (brand) bovine collagen (3.0 mg/ml). In an alternate embodiment, if heterotypic fibrils are being made for diameter control, type V collagen monomer is obtained as well. Extracted or recombinant human and or animal collagen type V monomers may be used.

Per step 220, collagen type I is prepared for polymerization. In a preferred embodiment, Vitrogen is neutralized in preparation for self-assembly by adding 8:1:1 ratio of collagen type 1:10x PBS: 0.1M NaOH. The pH is adjusted to 7.4. If type V is also included, the solution is further processed to ensure neutralization of type V as well. In preferred embodiments, additives may be used to alter the viscosity of the

collagen solution to change the shear stress on the growing fibrils. Such additives include, for example, but are not limited to, glycerol. The viscosity of the final solution of monomer is in the range of 1.0 mPa-s to 100.0 Pa-s. The preferred range is from 5.0 mPa-s to 1.0 Pa-s. In a preferred embodiment, the viscosity of the collagen monomer 5 solution is approximately 10.0 mPa-s.

The method 216 in accordance with the preferred embodiment includes the step 222 of substrate selection. The substrate that accepts the collagen coating may comprise of any material that can be generated with a uniform optically flat surface to promote the establishment of a uniform shear flow field during spin-coating. Asperities on the 10 surface of the material may be in a range of 0.0 to 10 microns, with a preferred range of 0.1 to .5 microns. A preferred embodiment, for example, utilizes a 2 inch diameter borosilicate glass disk.

The method 216 also includes the step 224 of preparation of the substrate to be coated with collagen. Preparation may include modulation of the surface of substrate to 15 be uniformly hydrophilic, uniformly hydrophobic, gradient hydrophilic, or to preferentially bind collagen, for example, antibody inclusion. A preferred embodiment for hydrophilic treatment for glass substrate utilizes, for example, 1.5 hour ultrasonication of substrate in 10% micro90 (brand) cleaner. Glass is stored in deionized water until use.

20 Per step 222, the substrate to be coated with collagen is positioned into the device designed to generate centripetal acceleration. In a preferred embodiment, the substrate is placed directly onto a vacuum chuck of a commercial spin-coater.

If multiple layers of collagen oriented at differing relative angles are desired another substrate handling system is required, for example, in a preferred embodiment a 25 borosilicate glass disk is placed in an offset disk holder of Figure 8 or a system as shown in Figure 4 is utilized.

The method 216 includes the step 228 of modulating the environment surrounding substrate to create conditions conducive to initiate polymerization of collagen. The environmental conditions include a temperature range of 30°C to 45°C.

with a preferred range of 35°C to 42°C and 80-100% humidity with a preferred range of 90-100% humidity. In a preferred embodiment, a steam heat humidifier is attached to vent ports in the housing of the substrate rotating device as shown in Figure 1. In alternate embodiments, the substrate or substrate holder may also incorporate a heating device to locally control temperature of the substrate surface. Such devices require 5 rotating electrical contacts.

A preferred embodiment of the present invention includes pre-processing or pre-wetting the substrate. Prior to sustained addition of the monomer to the substrate, a bolus of cold monomer is added to the substrate to ensure that the substrate is fully wet.

10 In a preferred embodiment, an amount capable of covering the entire substrate is injected and is used to be spread on the substrate.

The method 216 further includes step 230 of initiating a flow of the monomer units. Prior to substrate rotation, a flow of monomer is begun. For neutralized Nitrogen solution the start up range is 0.05-1000 ml/min with a preferred range of 0.1-100.0 ml/min. In a preferred embodiment, the start up flow rate for neutralized Nitrogen 15 solution is 2.0 ml/min.

The method then includes the step 232 of initiating rotation of the substrate. The rotation of substrate is initiated and may proceed in a series of steps to aid in the uniform spreading of the collagen monomer solution over the substrate surface. A range 20 of initial angular velocities during startup is 10 to 5000 rpm with a preferred range of 60 to 2000 rpm. In a preferred embodiment the initial angular velocity utilized is 250 rpm.

The method further includes the step 234 of controlling a plurality of parameters during polymerization. For example, the rotational velocity of the substrate during polymerization is set. During the polymerization of the collagen monomer on the warm 25 substrate, the rotational velocity may remain steady or undergo modulation of any kind. However, the average velocity (based on averaging over each minute of operation) of the substrate has a range of 100 to 50,000 rpm with a preferred range of 500 to 10,000 rpm. In a preferred embodiment, the average rotational velocity of the substrate is 1600 rpm.

Further, during polymerization, the flow rate of monomer is controlled. The monomer solution flow rate provides a monomer for polymerization to the substrate surface while generating suitable shear force under rotation to induce alignment of the growing collagen fibrils. The flow rate of collagen solution over the rotating substrate 5 may remain steady or undergo modulation of any kind. However, the average flow rate of collagen solution (based on averaging over each minute during operation) is in a range of between 0.05 ml/min to 1000 ml/min with a preferred range of 0.1 to 100 ml/min. In a preferred embodiment, the average flow rate over the substrate during polymerization is 0.25 to 2.0 ml/min.

10 Another parameter that is controlled during polymerization is the optimum shear rate at the substrate interface. The flow rate over the growing monomers tends to align them along the flow direction and provides the monomer units access to the free end. The combination of monomer solution input flow rate, viscosity and substrate rotational velocity combines to produce a range of shear rates from 1.0 to 500,000 Hz with a 15 preferred range of 50 to 50,000 Hz. In a preferred embodiment, the shear rate at the substrate surface is approximately 700 Hz.

The duration of polymerization is another parameter that can be controlled. Rheological experiments have demonstrated that the gelation of the collagen solution begins once optimum conditions are achieved and take approximately six minutes 20 following addition to a substrate warmed to 37°C. In the apparatus in accordance with a preferred embodiment of the present invention, the range of time of exposure of the monomer flow to the rotating substrate lies between 1 minute and 2 hours with a preferred range of 3 minutes to 1 hour. In a preferred embodiment, to generate a single layer of polymerized collagen, flow is sustained over the rotating substrate for 15 to 20 25 minutes.

In preferred embodiments, a layer including collagen type IV and cell adhesion proteins such as, for example, but not limited to, laminin, fibronectin and/or any integrin receptor is deposited between aligned polymer layers.

The method 216 in accordance with the present invention includes the step 236 of initiating a spin-down procedure. Following the addition of collagen to the rotating substrate, the rotating substrate continues spinning to remove excess collagen monomer. This spinning down procedure may include a rinse step where a solution containing no 5 collagen monomer is applied to the rotating substrate to enhance the removal of unreacted monomer or to add a layer of material to separate collagen layers, for example, to promote cell attachment and proliferation. Such materials may include proteoglycans, laminin, fibronectin and/or vinculin or integrin moieties. During the spin-down procedure the rotational velocity may remain steady or undergo modulation 10 of any kind. However, the average velocity (based on averaging over each minute of operation) of the substrate is in a range of 100 to 50,000 rpm with a preferred range of 500 to 10,000 rpm. In a preferred embodiment, the average rotational velocity of the substrate is 1600 rpm.

The method 216 concludes with a post-processing step 238. To ensure 15 polymerization of the deposited layer, the substrate may be post-processed. Post-processing may include an extended exposure to the warm humid environment for a period of 0.1 to 60 minutes with a preferred range of exposure of 3 to 10 minutes. In a preferred embodiment, the exposure time during post-processing is 5 minutes. The method 216 includes all of the essential steps to produce a single layer of aligned 20 collagen via spin-coating in accordance with a preferred embodiment of the present invention.

Figure 9 is a scanning electron microscope (SEM) image demonstrating the deposition of a plurality of thin aligned layers 244 onto a glass substrate 242 in accordance with a preferred embodiment of the present invention.

25 Figure 10 is a scanning electron microscope (SEM) image of a single layer of predominately aligned collagen fibrils in accordance with a preferred embodiment of the present invention.

Figure 11 is a scanning electron microscope (SEM) image of aligned collagen fibrils generated by the spin-coating methodology in accordance with a preferred embodiment of the present invention.

Figure 12 illustrates a scanning electron microscope (SEM) image demonstrating layering of collagen in pseudolamellae in accordance with a preferred embodiment of the present invention. Multiple layers of aligned collagen polymer may be achieved by repeating (indefinitely) the procedure for generating a single layer. The method 216 may be repeated immediately following the post-processing step or repeated following a dryout period or a deionized water soak period. In a preferred embodiment, multiple layers are achieved by repeating the single layer procedure following a 24 hour soaking period in deionized water.

Figure 13 is a SEM image 320 illustrating the intersection of two individual layers of aligned collagen in accordance with a preferred embodiment of the present invention. The arrows 322, 324 indicate the alignment directions for each layer. To generate multiple layers of aligned collagen polymer where the relative angle of alignment of the collagen is changed between depositions, the method 216 for producing multiple layers is performed. However, the substrate is rotated through any angle relative to its previous position on the substrate holder. Any range of angles is possible from a range of 0 to 2π (and any integer multiple thereof) with a preferred range of 0 to π . In a preferred embodiment, the angle between aligned collagen layers is $\pi/2$.

With regard to microfluidics methodologies, the fundamental principle in the embodiments of the present invention is to use flow regimes to control the growth of individual fibers of collagen, or some other polymerizable material. The method 216 outlined the use of a common industrial process, namely spin-coating, to provide the necessary constraints to the growing fibers to allow control of alignment, length and diameter. However in a more general embodiment it is possible to directly manipulate the flow field, and thus the local environment, around a growing filament. To this end the use of the emerging field of microfluidics can be used.

The use of microfabricated fluid handling is described by Giordano, N. and Cheng, J.-T. (2001) in the Journal of Physics: Condensed Matter, 13, R271-R295 entitled "Microfluid mechanics: progress and opportunities", the entire teachings of which is hereby incorporated by reference. These methods have matured over the last
5 few years resulting in commercial products such as DNA sorting systems. These systems can be used to handle fluids on sub-micron scales using features and channels that can be applied to sub-micron dimensions.

To control single growing filaments, the length scales required must be closer to the characteristic dimension of the filament, approximately 50 nm. Confinement in
10 tubes rapidly allows extension of the filament and the narrow confines of such a channel rapidly increase the shear rates around the filament. However these narrow channel sizes are currently difficult to manufacture and use. A solution is described by a preferred embodiment of the present invention and uses a form of flow focussing illustrated in Figures 14A-14C which provide a schematic of the flow focussing
15 concept. At the length scales discussed herein flow is almost always laminar. This means that in fact mixing is very difficult to encourage in such flow regimes. In reality then the mixing process is controlled by diffusion processes. Consequently if two jets impinge on each other at an angle they do not mix directly but must mix by diffusion
20 across the interface. If one of these jets contains the un-polymerized collagen (or other species) discussed hereinbefore, and the other jet contains the polymerizing agent then there is a finite time before enough diffusion occurs to allow mixing.

If the polymerizing jet has a higher volume flow rate and both jets enter the same size channel, then the amount of the channel used by each fluid "species" is proportional to the incoming flow rate. Consequently this behavior allows the fluids to
25 control the active zone in the flow field, both through constriction of the width as shown in Figure 14B, related to the relative flow rates (this is the flow focusing described hereinbefore), and through diffusion related to fluid concentrations.

The procedure outlined with respect to Figures 14A-14C allow a confined area to be generated in a flow field that can be arranged to have the correct solution

conditions for polymerization. In addition other fluids can be added to influence the filament diameter and, if necessary, stop polymerization. If the growing polymer is advanced such that the growing tip is always in this critical region, the polymer can be extruded indefinitely. This approach allows the nanofabrication of a single polymer.

5 Figure 15 illustrates another preferred system to manufacture layered aligned polymer structures in accordance with a preferred embodiment of the present invention. This preferred embodiment utilizes the recognition that if an array of channels such as described in Figures 14A-14C is manufactured an array of controlled polymers can be generated. In Figure 15, initially the collagen is polymerized against a fixed wall 384 to
10 ensure a dangling collagen chain is present in the nanoloom 386. As the collagen filament grows this nanoloom 386 advances relative to the fixed wall, thus extruding a single collagen filament as it advances. An array of these filaments allow construction of a single layer of aligned collagen. It may also be possible to produce woven materials in this manner.

15 Figures 16A and 16B illustrate a block diagram 420 and a schematic diagram 490, respectively, of a preferred embodiment system to manufacture layered, aligned polymer structures in accordance with the present invention. The apparatus 432 used to generate layered, aligned polymer structures has a plurality of input parameters 440 that can be modulated using a processor 480. The input parameters, include, without limitation, volume flow rate of monomer, temperature of monomer, monomer solution concentration, concentrations of monomer solution additives, substrate temperature, rotational velocity of substrate, inlet tube temperature, relative humidity of environment, and temperature of air. It should be noted that all parameters can vary with time. A plurality of output parameters 460 are also monitored and modulated by the processor 480. The output parameters include, without limitation, layer thickness, orientation of polymerizing layer, and radius of polymerized layer. The system includes a plurality of sensing elements and monitoring elements that enables the processor 480 to process, control and monitor different parameters. The processor through an input/output interface 442 interfaces with a pump to monitor and/or control the volume flow rate of

the monomer, the temperature of the monomer, the concentration of the monomer solution and the concentration of any additives to the solution. Further, the solution environment, for example, the temperature of the solution is monitored using the interface 424. The substrate environment, for example, the relative humidity and the 5 temperature can be monitored and modulated using an interface 426. The substrate conditions are monitored such as, for example, substrate temperature and the rotational velocity of the substrate. The parameters associated with the layers of aligned polymers, for example, layer thickness, orientation of polymerizing and radius of polymerized layers are also monitored using an interface 428. The processor 480 can have an 10 integrated display device or provide data to another display device and/or processor that is not co-located with the apparatus 432. Post-processing of the polymer structure as described with respect to the method illustrated in Figure 8B can be controlled by the processor 480. It should be noted that the device 432 can comprise a distribution network for the polymer solution that includes a plurality of nozzles that can be rotated 15 and deposit the polymer/monomer solution onto a substrate that can be stationary. The shear flow is generated by a relative motion and/or velocity between the distribution system and the substrate.

The system 420 may also include a microprocessor and a memory device that stores display data. The microprocessor may include an operating system, as well as 20 application and communication software to implement the functions with respect to controlling device 432 operation. The operating system for the system of the present invention includes a processing system with at least one high speed processing unit and a memory system. In accordance with the practice of persons skilled in the art of computer programming, the present invention has been described herein with reference 25 to acts and symbolic representations of operations or instructions that are performed by the processing system. Such acts, operations and instructions are also referred to sometimes as being computer executed or processing unit executed.

It will be appreciated that the acts and symbolically represented operations or instructions include the manipulation of electrical signals by the processing unit. An

electrical system with data bits causes a resulting transformation or reduction of the electrical signal representation, and the maintenance of data bits at a memory location in the memory system to thereby reconfigure or otherwise alter the processing unit's operation, as well as other processing of signals. The memory locations where data bits 5 are maintained are physical locations that have particular electrical, magnetic, optical, or organic properties corresponding to the data bits.

The data bits may also be maintained on a computer readable medium including magnetic disks, optical disks, organic disks, and any other volatile or non-volatile mass storage system readable by the processing unit. The computer readable medium 10 includes cooperating or interconnected computer readable media, which exist exclusively on the processing system or is distributed among multiple interconnected processing systems that may be local or remote to the processing system.

It should be understood that the programs, processes, methods and systems described herein are not related or limited to any particular type of computer or network 15 system (hardware or software), unless indicated otherwise. Various types of general purpose or specialized computer systems may be used with or perform operations in accordance with the teachings described herein.

In view of the wide variety of embodiments to which the principles of the present invention can be applied, it should be understood that the illustrated 20 embodiments are exemplary only, and should not be taken as limiting the scope of the present invention. For example, the steps of the flow diagrams may be taken in sequences other than those described, and more or fewer elements may be used in the block diagrams. While various elements of the preferred embodiments have been described as being implemented in software, other embodiments in hardware or 25 firmware implementations may alternatively be used, and vice-versa.

It will be apparent to those of ordinary skill in the art that methods involved in layered aligned polymer structures and methods of making same may be embodied in a computer program product that includes a computer usable medium. For example, such a computer usable medium can include a readable memory device, such as, a hard drive

device, a CD-ROM, a DVD-ROM, or a computer diskette, having computer readable program code segments stored thereon. The computer readable medium can also include a communications or transmission medium, such as, a bus or a communications link, either optical, wired, or wireless having program code segments carried thereon as 5 digital or analog data signals.

The systems and methods of the present invention can be used in a plurality of applications. For example, single layers of aligned collagen can be manufactured which can be used as a test bed for assessing effects of aligned collagen matrices on cellular behavior in connective tissue fibroblasts, in epithelia and in endothelia. Further they 10 can generate scaffolding to promote adhesion and proliferation of cell populations in corneal epithelium and/or corneal endothelium.

Further, these embodiments of the present invention can be used to generate multiple layers of aligned collagen which can be used as a test bed for examination of the behavior of cells in anisotropic extracellular matrix including cartilage fibroblasts, 15 corneal keratocytes, and tendon fibroblasts. They can generate connective tissue scaffolding for repair and promotion of cellular adhesion and proliferation that can be used in, for example, but not limited to, artificial corneal replacement, corneal repair material, transfer scaffolding for epithelial transplants, transfer scaffolding for endothelial transplants, tendon replacement or repair, ligament replacement or repair 20 and annulus fibrosis replacement or repair. In addition, biocompatible strengtheners for natural and/or artificial materials for use in tissue repair or replacement can be generated using the embodiments of the present invention. These can be rolled up to perform annulus fibrosis function while embedded in a poly(vinyl alcohol) matrix, or be used as strengtheners for corneas made from artificial materials, for example, poly HEMA 25 and/or be a resorbable anchor for tissue repair.

Further applications of the preferred embodiments include generating multiple layers of other aligned biopolymers that can be used as biocompatible scaffolding, for example, "braids" to strengthen stents, or other implants. They can also be used as guidance for nerves, for example, nerve cuffs. Other applications having layers where

alignment is not important that can benefit from the layered polymer structures of the present invention include support for species embedded in multiple layers such as cells and drug release applications.

In addition, applications that include non-biopolymers may benefit from the
5 deposition of layered, aligned polymer structures, for example, generation of optical storage media.

Example 6

The physical principles governing spin-coating flows are discussed in further detail below. Spin-coating is one of the simplest and most common approaches for
10 applying thin films to wafers and substrates. Thus, the behavior of fluids in this kind of configuration has been well studied. In its simplest form the fluid is undergoing quasi-solid body rotation on the disk and the only velocity components are rotational and radial. The fluid behavior is therefore controlled by the balance between the centripetal and viscous forces.

15 For the situation where there is a steady flow rate of fluid onto a spinning disk the solution requires an analysis of the full Navier-Stokes equation. Leneweit et al. (G. Leneweit, K. G. Roesner, R. Koehler, Experiments in Fluids 26, 75-85 (1999)) outlined work by Rauscher et al. (J. W. Rauscher, R. E. Kelly, J. D. Cole, Journal of Applied Mechanics 40, 43-47 (1973)) that generates a deceptively simple solution to the steady
20 flow problem. To first order, this solution is:

$$u = \frac{r\omega^2 h^2}{v} \left(\frac{z}{h} - \frac{1}{2} \left(\frac{z}{h} \right)^2 \right); h = \left(\frac{3}{2\pi} \frac{Qv}{\omega^2 r^2} \right)^{\frac{1}{3}} \quad \text{Equation 3}$$

where u is the radial velocity as a function of z away from the disk at a position r radially, h is the thickness of the film, ω the rotation rate of the disk, v is the kinematic viscosity of the solution and Q the volume flow rate. Calculations made using these relationships for practical conditions are summarized in Figures 17A and 17B.

25 Figure 17A is a graphical representation 500 of the relationships between position within the layer or layer thickness on the ordinate and radial position or

velocity on the abscissa for conditions of 750 rpm (dashed lines) and 1600 rpm (continuous lines) with constant addition of collagen solution (0.1 ml/min) to the center of a 2.5 cm radius disk. For both sets of flow conditions, the fluid layers are of the order of 10 μm at 25 mm from the point of deposition. In addition, from 10 mm to 25 mm
5 neither layer thickness varies by more than 10 μm . Figure 17B is a graphical representation 520 of the relationships between shear rate or velocity of fluid ordinate and normalized position in layer at 25 mm from center on the abscissa for conditions of 750 rpm (dashed lines) and 1600 rpm (continuous lines). Spin coating flow velocity profiles are semi-parabolic with high shear rates at the disk surface ($200-550 \text{ s}^{-1}$) and
10 moderate flow rates at the free surface (1.25 – 2 mm/sec). This analysis predicts that a virtually flat layer can be generated that will confine the growing collagen film and provide shear rates in excess of 100 s^{-1} near the substrate surface. These shear rates compare well with those seen experimentally in the work with DNA. Another critical parameter, the relaxation time for the growing collagen fibril, must vary with length of
15 the growing fibril.

Orthogonal stacking of multiple layers of aligned collagen fibrils was produced by placing a glass substrate off-center in a specially designed offset disk holder (Figure 8A). Collagen solution was applied at the center of rotation for the entire assembly as shown in Figure 8A. After one layer of collagen was applied, the glass substrate could
20 be rotated any arbitrary angle and another layer was applied. At the exact center of the glass substrate, the collagen layers cross each other at the same angle by which the glass substrate was rotated.

The surface profiles of the films generated are relatively flat to within 10 μm over the distance of 1.0 cm at the outer edge of the disk. At 2.5 cm from the center, the
25 film thickness is between 8.0 and 12.0 μm for the two flow regimes examined. Briefly, cold collagen solution was applied at 0.1 ml/min for 60 minutes to a warmed (37°C), centered 5 cm diameter glass disk, cleaned for 1 hour in Micro 90TM solution. To make isotonic, neutralized solutions of bovine collagen, VitrogenTM (Cohesion Technologies, BC) collagen solution, chilled to 4-6°C is mixed into 10x PBS in an 8:1 ratio. One part

0.1 M NaOH is then added. The pH of the solution is adjusted to 7.4 by the addition of 0.1 M HCl or 0.1M NaOH. The neutralized isotonic collagen solution is stored at 4-6°C for a maximum of 4 hours before use. If necessary, to increase the wetting ability of the collagen solution, Triton X100 surfactant (Sigma Aldrich, MO) is added (about 1 drop/20 ml collagen solution).

In a preferred embodiment, collagen films are made in a spin-coater (WS-400A-6NPP/LITE, Laurell Technologies) where the internal environment can be controlled to 25°C and 100% RH. Temperature control is suitably accomplished by addition of a PELT-1 and PTC-1 temperature controller (Sable Systems, NV) to the spin coater environmental chamber. Suitably, humidity can be controlled by attaching a DG-1 humidity controller (Sable Systems, NV) to the spin coater environmental chamber. Preferably collagen films are made, and solutions and substrates prepared, in a class 100 laminar flow clean hood (Air Science Technologies, FL) to prevent contamination by dust or other particulates.

The spin rate was 750 rpm and the chamber humidity was greater than RH=95%. Following the coating process the film was allowed to dry and then prepared for SEM. Figure 18 shows a SEM image 540 of fibril alignment 2.0 cm from the fluid deposition point; the flow rate was 0.25 ml/min and the rotation velocity was 1600 rpm. The top layer in this particular area formed incompletely due to de-wetting during the film formation, and the lower, orthogonal layer of fibrils can be seen passing under the top layer.

In general, improving wet-ability and preventing de-wetting can be accomplished by methods known in the art. Glass surfaces can be treated by hydrosilation (J. J. Pesek, M. T. Matyska, Interface Science 1997 5: 103-117). Hydrosilation is a robust approach that is slightly more complex than traditional silanization (J. J. Pesek, et al., Journal of Chromatography A 1998 818: 145-154). The natural hydroxide (Si-OH) group of the glass surface is silanized using a reactive silane agent. This surface is then reacted in the presence of a catalyst such as hexachloroplatinic acid and a CH₂=CH-R molecule which allows a vast array of

potential surface treatments. The effect of a given treatment can be assessed by the contact angle which can be determined using optical methods known in the art. See, for example, D. J. Shaw, Colloid and Surface Chemistry (Butterworth-Heinemann, Oxford, ed. Fourth, 1994). Exemplary treatments include those that can produce a contact angle
5 <30 ("hydrophilic" – e.g. Allyl glycidyl ether), those that can produce a contact angle
>120 ("hydrophobic" – e.g. 1-octene) and those that produce steric protection (e.g. poly
(ethylene oxide) based systems).

In some embodiments, the formed collagen films can be further modified with additives to promote cell attachment and proliferation, as noted above. Such additives
10 may include proteoglycans, laminin, fibronectin and/or vinculin or integrin moieties and mixtures thereof. Where the additive is a proteoglycan, the proteoglycan is suitably selected from the group consisting of decorin, lumican, biglycan, keratocan or syndican.

In some embodiments, the proteoglycan is decorin. Decorin represents nearly 40% of the total proteoglycan in the cornea and has been proposed to play an important
15 role in fibrillogenesis, tissue repair and the regulation of transforming growth factor-beta (TGF- β has a single chondroitin/dermatan sulfate chain near the N-terminus, and is subject to N-linked glycosylation). Decorin can bind to collagens, TGF- β , epidermal growth factor (EGF) receptors and fibronectin. In decorin-null mice, the collagen network in the skin is shown to be loosely packed with irregular contours and unnatural
20 lateral fusion of collagen fibrils is evident. Decorin core protein can be purified from tissue in a technique that preserves native structure and function (C. T. Brown et al., Protein Expr Purif 25, 389-99 (Aug, 2002). Unlike most procedures this method does not rely on the use of strong denaturing reagents that may compromise biological activity. For example, exhaustive extraction of 100 pulverized bovine corneal stromas
25 (99.7 g wet) yielded 225 mg of total GAG, 2.45 g of total protein and 110.8 mg total decorin. The final purification yielded 35 mg of decorin core protein per 100 corneas. This extraction consisted of a heterogeneous mixture of proteins and proteoglycans (CSPGs and KSPGs). Elution of the proteoglycans with 1.5 M NaCl resulted in 98% recovery of GAGs and 95% recovery of the decorin. The presence of decorin during

collagen fibrillogenesis prevents the lateral fusion of fibrils, which is apparently responsible for the reduction in the rate of fibrillogenesis reported in the literature. When used, decorin is present in the collagen monomer solution at the concentration of about 1 to about 100 $\mu\text{g}/\text{ml}$, suitably about 5 to about 50 $\mu\text{g}/\text{ml}$.

5 In some embodiments, the proteoglycan is lumican. It is known that mice homozygous for a null mutation in lumican (a keratan sulfate proteoglycan) do not have clear corneas. X-ray diffraction studies indicate that in these corneas the collagen network is in disarray and that there is a significant polydispersity in the diameter of the corneal collagen fibrils that may lead to the observed opacity.

10 **Example 7**

As another preferred embodiment of the material described in this invention, epithelial cells and endothelial cell layers are established in the structure generated in either of the first three examples with collagen to develop a corneal construct. This construct produces a cornea that is similar physiologically, ultrastructurally and 15 biochemically to normal mammalian corneas. The construct is suitable as a physiological or biomechanical model, and may find utility as a material for transplant into human subjects.

Tissue engineering has met with great success in producing artificial skin and is advancing rapidly in the development of artificial vessels. The cornea, being a fairly 20 simple, avascular tissue, is an attractive next milestone for such an approach. Indeed, artificial corneal constructs have been produced already by culturing corneal cells onto and within a collagen gel. Unfortunately, these constructs are not yet suitable for use in the clinic because the primary functions of the cornea (protection, refraction and transmission) are fundamentally dependent on the ultrastructural organization of the 25 stromal matrix. A stromal-centric approach is necessary. Tissue engineering alone has not been able to reproduce the stroma on the nanoscale because, to date, corneal fibroblasts cannot be induced to synthesize normal stromal architecture, nor can it be produced artificially, *de novo*. However, recent data show that activated human corneal keratocytes can produce the appropriate matrix components, but that they are not

organized. Stromal development is a complex event that depends on an intricate choreography of signaling, synthesis and contact guidance. In short, the cells need an organized matrix to direct the synthesis of additional extracellular matrix (referred to herein as “templating”). In preferred embodiment, biomimetic “primary” stromal

5 extracellular matrix (ECM) template provides adequate contact guidance to induce activated stromal keratocytes to produce organized “secondary stroma”. The present invention provides such a “primary” stromal template, *de novo*, by precisely controlling collagen fibril alignment on the nanoscale using microfluidics and collagen type I self-assembly as described in Examples 1-6 above. Such a primary stroma can be generated

10 either by orthogonally stacking these *de novo* manufactured lamellae, or by using a small number of them to “contact guide” human corneal keratocytes that have been induced to produce ECM.

Multilaminar collagen structures of the present invention can be used as nanostructure artificial templates (NAT) that are seeded with cells, such as fibroblasts, epithelial cells and endothelial cells to form artificial corneas. In preferred

15 embodiments, artificially produced nanostructured biomaterials are used as a template to induce the replication of corneal ultrastructure by fibroblast cells. In some embodiments, the cells are applied to unstretched NATs. In other embodiments, the cells are applied to NATs that are stretched to mimic physical environment of the cornea

20 *in situ*. The NAT can be simply constructed from collagen, or may also include additives such as proteoglycans, laminin, fibronectin and/or vinculin or integrin moieties. Where the additive is a proteoglycan, the proteoglycan is suitably selected from the group consisting of decorin, lumican, biglycan, keratocan or syndican.

Nanostructured materials derived from this process can be used to construct an artificial

25 corneal stroma for use in replacement or repair of corneal tissue. This templating approach requires the availability of an appropriate nanostructured artificial template (NAT) such as that described in Examples 1-6 above. In preferred embodiments, the NAT is a nanostructured artificial collagen template.

In one embodiment, the present invention provides a method comprising the steps of providing a nanostructured artificial template as a primary stroma; contacting the primary stroma with a first population of cells; and activating the first population of cells to form extracellular cellular matrix material on the nanostructured artificial

5 template to produce a secondary stroma, thereby producing a populated biomimetic corneal stroma. In some embodiments, the method further comprises the step of contacting a first surface of the populated biomimetic corneal stroma with corneal epithelial cells. In some embodiments, the method further comprises the step of contacting a second surface of the populated biomimetic corneal stroma with corneal

10 endothelial cells.

In the adult cornea, the stromal keratocytes exist in a quiescent state with a very low rate of cell proliferation (Zieske, J.D., et al., Kinetics of keratocyte proliferation in response to epithelial debridement. *Exp Eye Res*, 2001. 72(1):33-9). Upon corneal injury, the keratocytes are stimulated to proliferate and migrate to the wound site. The

15 activated keratocytes are termed fibroblasts. In some types of wounds, the fibroblasts differentiate further into myofibroblasts and exhibit filaments consisting of a-smooth muscle actin (Jester, J.V., et al., Expression of alpha-smooth muscle (alpha-SM) actin during corneal stromal wound healing. *Invest Ophthalmol Vis Sci*, 1995. 36(5):809-19; Masur, S.K., et al., Myofibroblasts differentiate from fibroblasts when plated at low

20 density. *Proc Natl Acad Sci U S A*, 1996. 93(9):4219-23). The fibroblasts and myofibroblasts also synthesize and secrete type I and III collagens, which are associated with scar formation (Nusgens, B.V., et al., Topically applied vitamin C enhances the mRNA level of collagens I and III, their processing enzymes and tissue inhibitor of matrix metalloproteinase 1 in the human dermis. *J Invest Dermatol*, 2001. 116(6):853-9;

25 Ohgoda, O., et al., Fibroblast-migration in a wound model of ascorbic acid-supplemented three-dimensional culture system: the effects of cytokines and malotilate, a new wound healing stimulant, on cell-migration. *J Dermatol Sci*, 1998. 17(2):123-31; Appling, W.D., et al., Synergistic enhancement of type I and III collagen production in

cultured fibroblasts by transforming growth factor-beta and ascorbate. FEBS Lett, 1989. 250(2):541-4). These cells also secrete a variety of other ECM components.

When keratocytes are isolated and placed into culture in the presence of serum or growth factors, they become proliferative and are most properly termed fibroblasts.

- 5 When these cells are grown in culture, they become increasingly quiescent as they reach confluence. However, in the early 1980's, several groups found that the addition of ascorbic acid (vitamin C) increased the proliferative rate of cultured fibroblasts (Tajima, S. and S.R. Pinnell, Regulation of collagen synthesis by ascorbic acid. Ascorbic acid increases type I procollagen mRNA. Biochem Biophys Res Commun, 1982. 106(2):632-7; Lyons, B.L. and R.I. Schwarz, Ascorbate stimulation of PAT cells causes an increase in transcription rates and a decrease in degradation rates of procollagen mRNA. Nucleic Acids Res, 1984. 12(5):2569-79.) In addition, it was reported that ascorbic acid stimulated the synthesis and secretion of ECM components such as type I and type III collagens (Nusgens, B.V., et al., 2001; Appling, W.D., et al., 1989; Hata, R. and H.
- 10 15 Senoo, L-ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissue-like substance by skin fibroblasts. J Cell Physiol, 1989. 138(1):8-16; Saika, S., et al., L-ascorbic acid 2-phosphate, a phosphate derivative of L-ascorbic acid, enhances the growth of cultured rabbit keratocytes. Graefes Arch Clin Exp Ophthalmol, 1991. 229(1): 79-83). Ascorbic acid acts as a cofactor for the enzymes responsible for hydroxylation of the lysine and proline residues on procollagen. These hydroxylations are required for the proper assembly of procollagen, which is secreted into the ECM. Subsequently, it was found that a more stable form of ascorbic acid, L-ascorbic acid 2-phosphate, has a far more potent effect on synthesis and secretion of ECM materials. L-ascorbic acid 2-phosphate was also found to stimulate the stratification of several fibroblast types, including dermal and corneal fibroblasts. This method of allowing the fibroblasts to assemble their own matrix has been used to engineer tissues including skin (Germain, L., et al., Can we produce a human corneal equivalent by tissue engineering? Prog Retin Eye Res, 2000. 19(5):497-527; Michel, M., et al., Characterization of a new tissue-engineered human

skin equivalent with hair. In Vitro Cell Dev Biol Anim, 1999. 35(6):318-26) and blood vessels (Germain, et al., 2000; Germain, L., M. et al., Tissue engineering of the vascular system: from capillaries to larger blood vessels. Med Biol Eng Comput, 2000.

38(2):232-40). It has been reported that a dermal substitute was reconstructed entirely
5 from cells grown in the presence of L-ascorbic acid 2-phosphate and including the addition of endothelial cells to generate capillary-like structures (Guido, S. and R.T. Tranquillo, Methodology for the systematic and quantitative study of cell contact guidance in oriented collagen gels. Journal of Cell Science, 1993. 105:317-331). In the blood vessel model, the fibroblasts assembled a dense ECM containing collagens,
10 glycosaminoglycans and elastin.

There is evidence that substrate surface structure can control cultured fibroblast morphology (Guido, S. and R.T. Tranquillo, Methodology for the systematic and quantitative study of cell contact guidance in oriented collagen gels. Journal of Cell Science, 1993. 105: 317-33), which in turn may control the structure of secreted matrix
15 in culture. (Wang, J.H., et al., Cell orientation determines the alignment of cell-produced collagenous matrix. J Biomech, 2003. 36(1): 97-102). This effect has been termed "contact guidance". In aligned collagen matrices, it has been reported that the depth of neurite elongation into the aligned media is far greater than for random matrix controls (Dubey, N., et al., Guided neurite elongation and Schwann cell invasion into
20 magnetically aligned collagen in simulated peripheral nerve regeneration. Exp Neurol, 1999. 158(2): 338-50). It has also been reported that nerve generation occurs more rapidly if axons are induced to traverse aligned collagen matrices rather than random collagen controls (Verdu, E., et al., Alignment of collagen and laminin-containing gels improve nerve regeneration within silicone tubes. Restor Neurol Neurosci, 2002. 20(5):
25 p. 169-79).

In addition to contact guidance, fibroblast and smooth muscle cell morphology, motion and differentiation may be influenced by strain or stretch of the substrate (Wang, J.H. and E.S. Grood, The strain magnitude and contact guidance determine orientation response of fibroblasts to cyclic substrate strains. Connect Tissue Res, 2000. 41(1): 29-

36; Girton, T.S., et al., Confined compression of a tissue-equivalent: collagen fibril and
cell alignment in response to anisotropic strain. J Biomech Eng, 2002. 124(5): 568-75;
Altman, G.H., et al., Cell differentiation by mechanical stress. FASEB J, 2002. 16(2): p.
270-2; Altman, G.H., et al., Advanced bioreactor with controlled application of multi-
5 dimensional strain for tissue engineering. J Biomech Eng, 2002. 124(6): 742-9).

Multilaminar aligned collagen structures are formed as described above and used
as nanostructure artificial templates (NAT). In some embodiments, the multilaminar
aligned collagen structures further comprise additives, such as proteoglycans. A
preferred proteoglycan is decorin. In some embodiments, the nanostructure artificial
10 templates are seeded with cells in a stress-free state. In other embodiments, strain is
applied to the nanostructure artificial template before being seeded with cells. Figure
19 schematically illustrates an Ussing-style perfusion chamber and a nanostructured
artificial template (NAT) comprising collagen placed in between the half-chambers and
a pressure is applied to apply strain to the structure. The perfusion chamber 600 has an
15 upper portion 620 and a lower portion 640 that enclose the nanostructure artificial
template 630. The upper portion 620 and a lower portion 640 are provided with
respective perfusion ports 624 and 644. Fluids can be applied to the perfusion ports,
and a pressure differential can apply stress to the nanostructure artificial template 630,
causing it to deform into, preferably, a curved shape.

20 In preferred embodiments, the nanostructured artificial collagen template is
seeded with fibroblasts and maintained in culture for a period before placing it in the
Ussing-style perfusion chamber. In preferred embodiments, the pressure placed on the
nanostructured artificial collagen template is selected to produce strains similar to
strains on actual *in vivo* corneal lamellae. In other embodiments, fibroblasts can be
25 applied to a pre-strained template.

Figures 20A-20C illustrate a flow chart for producing a cellular biomimetic
stroma from a nanostructured artificial template in accordance with a preferred
embodiment of the present invention. With reference to Figure 20A, the method 700
includes the step 710 of providing a nanostructured artificial template (NAT). In

general, the nanostructured artificial template is an aligned array of linear structures sufficient to induce a fibroblast to produce an aligned extracellular matrix which can in turn iteratively act to induce fibroblasts to produce subsequent aligned extracellular matrix. In preferred embodiments, the nanostructured artificial template is a

5 multilaminar aligned collagen structure formed as described above. Alternatively, a nanostructured artificial template can be constructed from other non-toxic materials such as non-collagen polymers, glass, metals or ceramics that can be structured into a linear array by photolithography, etching, molecular growth or other means suitable to produce a pattern of the required scale.

10 The method 700 also includes the step 712 of contacting the NAT with a first population of cells capable of iteratively producing an organized extracellular matrix array in response to the nanostructured artificial template. Suitable cells are eukaryotic cells. In a preferred embodiment, the eukaryotic cells are fibroblasts. In particularly preferred embodiments, the nanostructured artificial template comprises collagen and
15 the eukaryotic cells are mammalian fibroblasts. In some embodiments, the mammalian fibroblasts are derived from stimulated corneal stromal keratocytes.

In one preferred embodiment, stromal keratocytes harvested from human corneal donors are stimulated to produce extracellular matrix (ECM) using vitamin C (ascorbic acid) or a more stable derivative of vitamin C (L-ascorbic acid 2-phosphate, 20 pharmaceutically acceptable organic and inorganic acid addition salts thereof, preferably sodium ascorbyl phosphate or magnesium ascorbyl phosphate) and cultured onto the surface of the nanostructured artificial collagen template. The culture with the stimulated fibroblastic cells is maintained for a period of time and then the activity of the corneal fibroblasts is arrested by replacing the vitamin C in the perfusate with fetal
25 bovine serum, thereby arresting the fibroblastic activity of the cells and restoring them to their quiescent keratocyte phenotype. This procedure can produce a three-dimensional extracellular matrix that reflects and incorporates the structure of the nanostructured artificial collagen template. Cellular secretion of extracellular matrix (ECM) in the nanostructured artificial collagen template produces a “templated ECM (tECM)”.

Depending on the amount of organized matrix produced by the fibroblasts, the tECM may be used alone or may be stacked onto other such constructs and then used in the repair or replacement of diseased or damaged corneal stromal tissue. In addition, single or multiple tECMs may be used as a substrate onto which corneal epithelium and/or

5 endothelium may be cultured to produce a functional artificial cornea.

Cell harvesting procedure. Corneal buttons are removed from the central cornea using an 8-mm trephine. The corneal buttons are placed in Dulbecco's minimal essential medium (DMEM) plus antibiotics and cut into quarters. The quartered buttons are then placed in 21 ml of DMEM containing 3.3mg/ml collagenase (type L.C8170; Sigma

10 Aldrich, MO) and incubated at 37 ° C with shaking for 30 min. The tissue plus solution is then vortexed for 30 seconds and the tissue removed using a cell strainer. Collagenase digestion is repeated for 60 minutes, the tissue strained, and then a third repeat of 180 minutes. The cells in each of the three collagenase digestions are be collected by low speed centrifugation and resuspended in DMEM. Our studies have shown that the cells

15 from the third digestion represent the purest population of keratocytes. Cell numbers are determined using a hemocytometer and the cells are resuspended in DMEM containing 1% platelet-poor horse serum (Sigma Aldrich, MO) and plated in uncoated culture dishes. Culture medium is changed every 2-3 days.

The method 700 also includes the step 714 of maintaining the NAT with a first

20 population of cells in culture to produce a templated extracellular matrix.

Culturing cells onto NAT. In a preferred embodiment, nanostructured artificial collagen templates in PBS are placed onto the polycarbonate membrane (Costar, Charlotte, NC) of a 12 mm diameter Transwell™ insert (for unstretched protocol) or clamped into the pressurized perfusion chamber (for stretched protocol). The stromal

25 lamellae are allowed to equilibrate and settle overnight prior to seeding. Human stromal fibroblasts (2×10^6) are added to the Transwell™ in DMEM containing 10% FBS and 1mM L-ascorbic acid 2-phosphate. The lamellae and fibroblasts are incubated at 37°C and medium is changed every other day. For controls, the Transwell™ membrane alone is the substrate for the cell culture. The process of creating one layer of secreted matrix

is extended to generate a full-thickness stroma (450 μm). The number of stromal templates, n, is chosen such that the final thickness of the secreted matrix alone multiplied by n equals $\sim 450 \mu\text{m}$. The mounted stromal templates is seeded with activated human keratocytes covered with a second template matrix and cultured until 5 they have generated the anticipated amount of stromal tissue. At this time, the perfusion solution is switched to arrest matrix production and to promote the differentiation to quiescent keratocytes. The constructs are removed from their mounts and, if possible, the templates are dissected away. The remaining secreted stromal matrices are stacked to generate a thick stromal construct. The constructs are transferred to perfusion 10 chambers and loaded (stretched) for a period of up to 8 weeks. The stacks of *de novo* lamellae are populated with keratocytes to generate an appropriate stromal analog. Unlike stromas made via templating, the keratocytes in this construct are not activated to produce matrix, and express the quiescent keratocyte phenotype. The following seeding method is used to facilitate population of the *de novo* matrix with keratocytes., 15 Following the generation of thick stroma, the construct is placed onto a TranswellTM culture dish and pinned down lightly with a 1.0 cm O-ring (allowing the outer edges of the construct to swell and fray). Isolated human keratocytes and medium are added to the TranswellTM culture dish and allowed to infiltrate the edges of the construct for 24 hours. At the end of that time, a 1.4 cm O-ring is used to clamp the edges of the 20 construct and the 1.0 cm O-ring will be removed. Further infiltration of the construct by the keratocytes can be facilitated by maintaining the construct in this configuration until keratocytes reach the center (as determined by optical microscopy). Following infiltration of the keratocytes, the construct is transferred to the perfusion chamber and loaded (stretched) for a period of up to eight weeks. Confocal microscopy is used to 25 assess the infiltration of the keratocytes into the center of the stromal construct. The resulting cultured tissue can be harvested at 1, 4 and 8 weeks for light microscopy, TEM, QFDE and immunohistochemistry. A successful construct contains a stable population of quiescent keratocytes with few fibroblasts or myofibroblasts. The cells should exhibit an elongated flat morphology and reside between adjacent lamellae. The

construct ultrastructure should comprise multiple layers of aligned type 1 collagen with decorin distributed evenly throughout. Fibroblasts are found to populate the nanostructured artificial template and to secrete additional extracellular matrix.

The method 700 also includes the step 718 of deciding whether an additional 5 population of cells should be added to the templated extracellular matrix. If the answer is “no”, the process is ended 750. Otherwise, the method 700 is continued in method 720.

With reference to Figure 20B, the method 720 includes the step 722 of providing a templated extracellular matrix, such as that produced by step 714. Method 720 also 10 includes the step 724 of contacting a first surface of the templated extracellular matrix with a second population of cells. Suitable cells are eukaryotic cells.

In preferred embodiments, the second population of cells is a population of mammalian corneal epithelial cells. The corneal epithelium is a multicellular “tight” stratified squamous epithelium comprising three distinct functional layers. It is 15 approximately 50 microns thick in humans. The deepest cellular layer is the stratum germinatum, the only layer capable of undergoing mitosis. The middle layer comprises the daughter cells (wing cells) of the basal layer which are pushed anteriorly. The surface layer comprises the squamous cells that form the complete tight junctions which generate the primary barrier to transport in the cornea and are critical to understanding 20 its vegetative physiology. As the leading edge of the tough ocular tunic, the epithelial cells serve to protect and defend the underlying corneal stromal tissue. In constant state of turnover, the epithelial cells continually renew the corneal surface. The complete tight junctions, maintained during the turnover process, limit fluid transport and prevent infection. Active chloride transporters give the epithelium a limited ability to move fluid 25 out of the cornea to the tear film. However, physiologically, the epithelium is primarily a barrier. Following injury, epithelial cells rapidly re-cover the affected area and restore corneal clarity. It is becoming clear that interaction between the epithelial cells and underlying stromal cells is necessary to fully respond to injury.

The templated extracellular matrix may be stressed or unstressed. In preferred embodiments, the templated extracellular matrix is stressed into a curved form, and the first surface is the convex face of the curved templated extracellular matrix.

5 The method 720 also includes the step 726 of maintaining the templated extracellular matrix with a second population of cells in culture to produce a templated extracellular matrix having a layer of the second population of cells on the first surface.

In one embodiment, the second population of cells is added to the multilaminar templated extracellular matrix as follows. The methodology for the isolation and cultivation of human corneal epithelial cells is known in the art. In brief, the limbal ring 10 of the cornea is incubated 18-24 hours in a dispase solution at 2-8°C. The epithelium is separated and treated briefly with trypsin-EDTA (Life Technologies, Inc, MD) solution to dissociate the cells. The trypsin action is stopped by the addition of Trypsin Neutralizing Solution (Clonetics, MD); the cells are pelleted, resuspended in Keratinocyte-SFM medium (Life Technologies, Inc.), and seeded onto T-75 tissue 15 culture flasks coated with fibronectin-collagen (FNC) coating mix (Biological Research, MD). We routinely obtain one to two T-75 flasks of cells per cornea using this methodology (2×10^6 cells/T-75 flask). The limbal rings are from tissue used for corneal transplantation that would otherwise be discarded. Human corneas deemed unsatisfactory for transplant can also be obtained from NDRI.

20 A corneal epithelium cultured atop a corneal keratocyte-assembled templated extracellular matrix can be obtained as follows. After stabilization of the stromal construct, the media is removed from the construct surface and the construct is coated with FNC for one minute to promote adhesion of epithelial cells. The FNC is then removed and a suspension of corneal epithelial cells (2×10^5 /well) is added to the 25 coated construct. After the epithelial cells are seeded onto the matrix, Keratinocyte-SFM media is added to the anterior side of the construct and it is incubated at 37°C. After 24 hours, medium is added to the posterior side of the construct as needed. The cultures are maintained for an additional 24 hours (two days total), after which all media is removed. Fresh Keratinocyte-SFM medium supplemented with 0.3% FBS and 1.7 mM Ca++

(1.73 mM final concentration) is added to the posterior side only, thus causing the epithelium to be air lifted and maintained with a minimal covering of media. Calcium and FBS are added to stimulate epithelial stratification and differentiation. The cultures are maintained for one to two weeks, with media being changed three times per week.

5 The method 720 also includes the step 728 of deciding whether an additional population of cells should be added to the templated extracellular matrix having a layer of the second population of cells on the first surface. If the answer is "no", the process is ended 750. Otherwise, the method 720 is continued in method 730.

With reference to Figure 20C, the method 730 includes the step 732 of providing
10 a templated extracellular matrix having a layer of the second population of cells on the first surface, such as that produced by step 726. Method 730 also includes the step 734 of contacting the second surface of the templated extracellular matrix with a third population of cells. Suitable cells are eukaryotic cells.

In preferred embodiments, the third population of cells is a population of
15 mammalian corneal endothelial cells. Corneal endothelium is a single layer of tissue that forms a boundary between the corneal stroma and the anterior chamber. The endothelium from young individuals consists of polygonal-shaped cells, 4-6 mm thick with a diameter of around 20 mm. Corneal endothelial cells express occludin, an integral membrane protein associated with tight junctions, and ZO-1, a member of a
20 submembranous cytoplasmic complex associated with tight junctions. Abundant mitochondria indicate the high metabolic activity of these cells and are consistent with their role as the major fluid transporting layer in the cornea. The basal (anterior-most) aspect of corneal endothelial cells rests on Descemet's membrane, the thick basement membrane that is secreted by the endothelium. Proteins, such as vinculin, talin, b3-integrin, and alpha-v, beta-5 integrin are expressed in corneal endothelial cells,
25 suggesting that they form structures that facilitate normal cell-substrate adhesion. The primary function of the endothelium is to maintain corneal transparency by regulating corneal hydration against a large swelling pressure. Proteoglycans associated with stromal collagens bind water and produce a pressure gradient across the endothelium.

The endothelium counteracts the tendency of the corneal stroma to swell by removing excess stromal fluid via the activity of Na⁺/K⁺-ATPase and bicarbonate-dependent Mg²⁺-ATPase.

5 The templated extracellular matrix may be stressed or unstressed. In preferred embodiments, the templated extracellular matrix is stressed into a curved form, and the second surface is the concave face of the curved templated extracellular matrix.

The method 730 also includes the step 736 of maintaining the templated extracellular matrix with a third population of cells in culture to produce a templated extracellular matrix having a layer of the third population of cells on the second surface.

10 Methods of human corneal endothelial cell culture are disclosed in U.S. Patent No. 6,548,059, which is hereby incorporated by reference in its entirety. In preferred embodiments, donor corneas stored in Optisol-GS at 4°C are obtained from NDRI. For culturing of endothelial cells, corneas are placed in a petri dish containing Medium 199 and 50 µg/ml gentamicin. Under a dissecting microscope, Descemet's membrane with 15 the attached endothelium is stripped from the stroma and placed in a 15 ml centrifuge tube containing 0.2 mg/ml EDTA in Hank's BSS, pH 7.4. The tissue is incubated for 1 hour at 37°C, then cells are detached from Descemet's membrane by vigorous disruption with a flame-polished pipette. Cells are pelleted and then re-suspended in culture medium containing OPTIMEM-1 culture medium supplemented with 8% FBS, 20 40 ng/ml FGF, 5 ng/ml EGF, 20 ng/ml NGF, 20 µg/ml ascorbic acid, 0.005% human lipids, 200 mg/L calcium chloride, 0.08% chondroitin sulfate, 1% RPMI-1640 multiple vitamin solution, 50 µg/ml gentamicin, and antibiotic/antimycotic solution (1/100).

Cells are incubated in six-well tissue culture plates at 37°C in 5% CO₂, and medium is changed every other day. With this methodology, cells reach confluence in 10 to 14 days, after which time they will be subcultured and seeded at a split ratio of 1:4 or 1:8. Only cells from primary culture or passage one are used. Stromal matrix constructs (multilaminar templated extracellular matrix) are washed with growth medium prior to seeding cells directly onto the matrix. The stromal surface can also be pre-coated with fibronectin, laminin, or type IV collagen alone or in combination prior to cell seeding to

facilitate endothelial cell adhesion. The cell-matrix complex is then incubated, endothelial side-up, at 37 degrees Celsius in a 5% CO₂, humidified chamber for various periods of time.

The method 730 also includes the step 738 of deciding whether an additional 5 population of cells should be added to the templated extracellular matrix now having a layer of the second population of cells on the first surface and a layer of the third population of cells on the second surface. If the answer is "no", the process is ended 750. Otherwise, the method 730 is continued by repeating method 720 or method 730 as needed.

Figure 21A is a schematic diagram illustrating a flow chamber 800 for generating layered aligned polymer in accordance with a preferred embodiment of present invention. A preferred embodiment provides a method to generate single or multiple layers of aligned polymer fibrils by pumping monomer solution between two surfaces, with an adjustable gap between them. The flow chamber 800 is lined by a collagen accepting layer 822 and a collagen rejecting layer 820 that, together with inlet 810 and outlet 812, define a space in which collagen polymerizes. The collagen rejecting surface 820 is attached to an upper thermoelectric device 822; the collagen accepting surface 822 is attached to a lower thermoelectric device 820. The upper and lower thermoelectric devices are preferably provided with heat sinks that may be solid or that may contain channels through which cooling fluids may be circulated. The height 828 is adjustable using a micromanipulator 830 that is attached to the upper thermoelectric device 822.

A collagen accepting surface 822 may be generated by coating the surface may be generated by coating the surface with antibodies to collagen, by plasma cleaning, by cleaning with micro 90, by functionalization, or by treating the surface with any methods known in the art to attract and promote adherence of collagen monomer or polymer units. A collagen rejecting surface 820 may be generated by functionalization, surface treatments, coatings or use of materials known in the art to limit, reduce, or reject the adhesion of collagen monomer or polymer units.

Single or multiple layers of collagen can be produced by regulating collagen self assembly conditions in the space bordered by the surfaces 820, 824 and inlet 810 and outlet 812. In preferred embodiments, the control of parameters such as flow rate and temperature are monitored and adjusted under computer system control, as described
5 above.

In a preferred embodiment, the collagen monomer solution 804, as described above, enters the chamber through the inlet 810. The temperature of the surfaces 820 and 824 is regulated by the associated upper thermoelectric device 822 and lower thermoelectric device 826. In a preferred embodiment the upper thermoelectric device
10 822 is operated to cool the collagen rejecting surface 820 and the lower thermoelectric device 826 is operated to heat the collagen accepting surface 824.

Referring to Figure 21B, in a preferred embodiment, layers of collagen in which the fibrils are aligned substantially orthogonal to the adjacent layers are formed by use of inlet 810 and corresponding outlet 812 alternating with use of inlet 814 and
15 corresponding outlet 816. After the formation of a layer of aligned collagen fibrils in one direction, using inlet 810 and outlet 812, the micromanipulator 830 is used to increase the height 828 to provide space for an additional layer, and collagen monomer solution then flows from inlet 814 to outlet 816. Further iterations produce a multilaminar nanostructured artificial template. The input collagen monomer solution
20 804 is as described above. The outflow 808 is reduced in monomer concentration. Depending on flow rate and other parameters, the outflow 808 may be refluxed through the chamber 800 to improve the efficiency of use of monomer solution.

The claims should not be read as limited to the described order or elements unless stated to that effect. Therefore, all embodiments that come within the scope and
25 spirit of the following claims and equivalents thereto are claimed as the invention.